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U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
RUBINSTEIN=7

U S APPLICATION NO (If known,see 37 CFR 1 5)
10/070295

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY CLAIMED
PCT/IL00/00525	04 September 2000	05 September 1999

TITLE OF INVENTION
USE OF LEPTIN IN INHIBITION OF ENDOTHELIAL CELL PROLIFERATION

APPLICANT(S) FOR DO/EO/US
Menahem RUBINSTEIN et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U S.C 371.
3. [X] This is an express request to begin national examination procedures (35 U S.C 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S C 371(b) and PCT Articles 22 and 39(1).
4. [X] The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31)
5. [X] A copy of the International Application as filed (35 U.S C. 371(c)(2))
   a. [ ] is attached hereto (required only if not transmitted by the International Bureau).
   b. [X] has been communicated by the International Bureau
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] An English language translation of the International Application as filed (35 U S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been communicated by the International Bureau
   c. [ ] have not been made, however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U S C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S C. 371(c)(4))
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. [ ] An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3 31 is included.
13. [ ] A FIRST preliminary amendment
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    [X] Courtesy copy of the International Application as filed
    [X] Courtesy copy of the first page of the International Publication (WO 01/18040)
    [X] Courtesy copy of the International Preliminary Examination Report. There were no annexes.
    [X] Formal drawings, 7 sheets, Figures 1-7.
    [X] Courtesy Copy of the International Search Report.
    [X] Application Data Sheet
    [X] The application is (or will be) assigned to: Yeda Research and Development Co. Ltd , whose address is Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot, Israel.

U.S. APPLICATION NO (If known, see 37 CFR 1.5)	International Application No	Attorney's Docket No
10/076295	PCT/IL00/00525	RUBINSTEIN=7

17. [xx] The following fees are submitted.

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO... ... ... **$1040.00**		
International preliminary examination fee (37 CFR 1 482) not paid to USPTO but International Search Report prepared by the EPO or JPO... ..... **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1 445(a)(2)) paid to USPTO.. . ...**$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1 482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... . **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... ....**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims as Originally Presented	Number Filed	Number Extra	Rate		
Total Claims	10 - 20		X $18.00	$	
Independent Claims	3 - 3		X $84.00	$	
Multiple Dependent Claims (if applicable)			+$280.00	$ 280.00	
TOTAL OF ABOVE CALCULATIONS =				$1,300.00	

Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate		
Total Claims	- 20		X $18.00	$	
Independent Claims	- 3		X $84.00	$	
TOTAL OF ABOVE CALCULATIONS =				$1,300.00	

Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27	$	
SUBTOTAL =	$1,300.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1 492(f)).	$	
TOTAL NATIONAL FEE =	$1,300.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3 31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$1,300.00	
	Amount to be: refunded	$
	charged	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed
b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of $ 1,300.00, is attached.
c. [ ] Please charge my Deposit Account No. **02-4035** in the amount of $_____ to cover the above fees.
A duplicate copy of this sheet is enclosed
d. **[XX]** The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4035**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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REGISTRATION NUMBER

USE OF LEPTIN IN INHIBITION OF ENDOTHELIAL CELL PROLIFERATION

Field of the Invention

The present invention relates to reversible inhibition of endothelial cell proliferation and to modulation of angiogenesis in the female reproductive system. More particularly, the present invention relates to the use of leptin or leptin homologues or derivatives, optionally together with inhibitors of VEGF action or inhibitors of VEGF synthesis, in the preparation of a medicament for inhibiting angiogenesis or modulating angiogenic processes. The present invention further relates to the use of leptin or leptin homologues or derivatives, together with inhibitors of VEGF action or inhibitors of VEGF synthesis, in the preparation of a medicament for modulation of angiogenesis in the female reproductive system.

Background of the Invention

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development, formation of the corpus luteum, endometrium and placenta and growth of adipose tissue.

The term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels, and blood vessels. Both controlled and

uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes, surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the pathological damage seen in these conditions. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic dependent or angiogenic associated diseases. The hypothesis that tumor growth is angiogenesis-dependent was first proposed in 1971. (Folkman J., Tumor angiogenesis: Therapeutic implications. N. Engl. J. Med. 285:1182-1186, 1971). In its simplest terms it states: "Once tumor 'take' has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries converging on the tumor." Tumor 'take' is currently understood to indicate a prevascular phase of tumor growth in which a population of tumor cells occupying a few cubic millimeters volume and not exceeding a few million cells, can survive on existing host microvessels. Expansion of tumor volume beyond this phase requires the induction of new capillary blood vessels. For example, pulmonary micrometastases in the early prevascular phase in mice would be undetectable except by high power

microscopy on histological sections.

Vascular morphogenesis is regulated by the hypoxia-induced vascular endothelial growth factor (VEGF) and its endothelial cell receptors Flk1 and Flt1. Two other angiogenic factors, angiopoietin-1 and 2 (Ang1 and Ang2), which bind to a common endothelial cell receptor (Tie2), were identified (S. Davis, et al., *Cell* **87**, 1161-1169, 1996; P. C. Maisonpierre, et al., *Science* **277**, 55-60, 1997). Ang1 is a receptor agonist (C. Suri, et al., *Science* **282**, 468-471, 1998), constitutively expressed in many tissues, whereas Ang2 is a receptor antagonist, whose expression is limited to sites of vascular remodeling. So far, Ang2 was identified in fetal tissues, in endothelial cells, in smooth muscle cells and in female reproductive organs of adult mice and humans (P. C. Maisonpierre, et al., *Science* **277**, 55-60, 1997; B. Witzenbichler, P. C. Maisonpierre, P. Jones, G. D. Yancopoulos, J. M. Isner, *J Biol Chem* **273**, 18514-18521, 1998; S. J. Mandriota, M. S. Pepper, *Circ Res* **83**, 852-859, 1998). Both VEGF and Ang2 are up-regulated in female reproductive organs upon vascular morphogenesis, whereas only Ang2 is expressed upon blood vessel regression. Ang2 probably marks these vessels for regression by an apoptotic mechanism, although induction of apoptosis by Ang2 in cultured endothelial cells has not been obtained (B. Witzenbichler, P. C. Maisonpierre, P. Jones, G. D. Yancopoulos, J. M. Isner, *J Biol Chem* **273**, 18514-18521, 1998; J. Holash, et al., *Science* **284**, 1994-1998, 1999; D. Hanahan, *Science* **277**, 48-50, 1997).

A specific antibody against VEGF reduces microvessel density and causes "significant or dramatic" inhibition of growth of three human tumors, which rely on VEGF as their sole mediator of angiogenesis (in nude mice). The antibody does not inhibit growth of the tumor cells in vitro. (Kim K J, et al., Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor

growth in vivo. Nature 362:841-844, 1993).

A specific angiogenesis inhibitor (AGM-1470) inhibits tumor growth and metastases in vivo, but is much less active in inhibiting tumor cell proliferation in vitro. It inhibits vascular endothelial cell proliferation half-maximally at 4 logs lower concentration than it inhibits tumor cell proliferation. (Ingber D, et al., Angioinhibins: Synthetic analogues of fumagillin which inhibit angiogenesis and suppress tumor growth. Nature, 48:555-557, 1990). There is also indirect clinical evidence that tumor growth is angiogenesis dependent.

Adipose tissue microcirculation is unique within the vascular system because of the capacity of this system to grow throughout most of adult life (D. L. Crandall, G. J. Hausman, J. G. Kral, *Microcirculation* **4**, 211-232, 1997). Indeed, brown and white adipose tissues have an extensive microvasculature and express high levels of VEGF (K. P. Claffey, W. O. Wilkison, B. M. Spiegelman, *J Biol Chem* **267**, 16317-16322, 1992; Q. X. Zhang, et al., *J Surg Res* **67**, 147-154, 1997). Thus, it is clear that angiogenesis plays a major role in the growth and maintenance of adipose tissue. If this angiogenic activity could be repressed or eliminated, then the adipose tissue will regress.

Obesity, defined as an excess of body fat relative to lean body mass, is associated with important psychological and medical morbidities, the latter including hypertension, elevated blood lipids, and Type II or non-insulin-dependent diabetes mellitus (NIDDM). There are 6-10 million individuals with NIDDM in the U.S., including 18% of the population of 65 years of age (Hanis et al., Ira. J. Obes., 11:275-283, 1987). Approximately 45 % of males and 70% of females with NIDDM are obese, and their diabetes is substantially improved or eliminated by weight reduction (Harris, Diabetes Care, 14(3):639-648, 1991).

Y. Zhang et al (Nature, 372, 425-431, 1994) suggest that one of the molecules which plays a key role in energy balance regulation is the ob protein also termed leptin. Zhang et al also report the cloning and sequencing of both mouse and human leptin. United Kingdom patent specification No. 2292382 relates inter alia to polypeptides, ob polypeptides or allelic variants or analogs thereof and their use for modulating body weight. In particular, GB 2292382 discloses that leptins and certain analogs thereof, such as agonists, would be useful for the treatment of obesity. Indeed, it was found that the adipocyte-derived leptin regulates food intake in rodents through its action on an hypothalamic receptor. Yet, later studies have shown that serum leptin is elevated in obese individuals and that there is a direct correlation between serum leptin and the body mass index (weight in kg divided by squared height in m.). The discrepancy between leptin's effect as an inhibitor of food intake and the high levels of leptin in obese individuals led to the theory of "leptin resistance", a term suggesting that obese individuals do not respond to their high leptin levels and maintain their high body mass. Thus it is clear that leptin by itself is not efficient in reducing the adipose tissue mass (P. Prolo, M. L. Wong, J. Licinio, *Int J Biochem Cell Biol* **30**, 1285-1290, 1998).

There exists therefore a need for a composition and method which can inhibit the unwanted growth of blood vessels, especially into tumors and adipose tissues. The composition should also be able to modulate the formation of capillaries in other angiogenic processes, such as wound healing and reproduction. The composition and method for inhibiting angiogenesis should preferably be non-toxic and produce few side effects. If angiogenic activity could be repressed or eliminated, then tumor, although present, would not grow and adipose tissue will regress. In the disease state, prevention of angiogenesis could

avert the damage caused by the invasion of the new microvascular system. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

Mice lacking leptin are infertile because leptin is required for release of gonadotropin-releasing hormone (GN-RH) from the hypothalamus. GN-RH acts on the pituitary gland and is essential for the release of the gonadotropins FSH and LH. Indeed, injection of leptin to leptin-defficient mice resuced their sterility. Feamales who have low adipose tissue mass, as the case of athletes or anorexia nervosa patients are infertile due to insufficient level of the adipose tissue-produced leptin.

One of the characteristics of the estrous cycle is ovarian angiogenesis, which takes place during the maturation of the follicle in the ovary. Rupture of the follicle and formation of the corpus luteum are associated with extensive blood vessel regression. These tissues were shown to express VEGF and Ang2. Therapies directed at control of angiogenic processes in the female reproductive system could regulate fertility.

Summary of the Invention

The present invention relates to the use of leptin, a leptin homologue or a derivative thereof, optionally together with an inhibitor of VEGF action or of VEGF synthesis, in the preparation of a medicament reversibly inhibiting endothelial cell proliferation.

In one aspect, the invention relates to the use of leptin, a leptin homologue or a derivative thereof in the preparation of a medicament for modulating angiogenic processes.

More particularly, the use in inhibition of angiogenisis is contemplated.

In another aspect, the invention provides for the use of leptin, a leptin homologue or a derivative thereof together with an inhibitor of VEGF action or VEGF synthesis in the preparation of a medicament for regulating fertility in a mammal.

Any known pharmaceutically acceptable VEGF inhibitor may be employed in accordance with the invention.

The invention also relates to pharmaceutical compositions modulating angiogenic processes or body weight or fertility comprising leptin, a leptin homologue or a leptin derivative optionally together with an inhibitor of VEGF action or VEGF synthesis.

Preferably the composition is employed in angiogenesis mediated diseases.

Brief Description of the Figures

Figure 1 shows a leptin induced blood vessel regression and apoptosis in adipose tissues of C57BL-*ob*$^{-/-}$ mice. C57CB-*ob*$^{-/-}$ mice were injected with murine leptin (2x1 μg/g) at time 0 and 9 h. Abdominal adipose tissues were removed at 24 and 48 h. Blood vessels were visualized in tissue sections by immunostaining with antibodies to Factor VIII (DAKO A/S, Denmark). Note that the number of stained blood vessels has decreased in 24 and 48 h post injection.

Figure 2 shows a dose-response curve of the blood vessel regression in adipose tissue of *ob*$^{-/-}$ mice 24 and 48 h post leptin injection.

Figure 3 shows a time course of the blood vessel regression in adipose tissues of *ob*$^{-/-}$ mice injected with murine leptin (2x1 μg/g).

Figure 4 shows a leptin-mediated induction of angiopoietin-2 (Ang2) as analyzed by reverse transcription-PCR of RNA from adipose tissues. Lane 1, control (no RNA); lane 2, RNA of adipose tissue from normal C57BL mouse; lane 3, RNA of adipose tissue from C57BL mouse injected with leptin (2x5 μg/g) lane 4, RNA of adipose tissue from C57BL-*ob*$^{-/-}$ mouse; lane 5, RNA of adipose tissue from C57BL-*ob*$^{-/-}$ mouse injected with leptin (2x5 μg/g). PCR reactions were terminated before saturation. PCR primers of Ang2, GeneBank Accession no. AF004326, corresponded to positions 637-657 (sense) and 1167-1147 (reverse).

Figure 5 shows a time course of Ang2 induction by leptin in adipose tissue of *ob*$^{-/-}$ mice. Leptin (2x5 μg/g) was administered to C57BL-*ob*$^{-/-}$ mice, total adipose RNA was extracted at the indicated times and analyzed by RNA blotting with probes to mouse Ang2, VEGF and actin.

Figure 6 shows a dose response of Ang2 induction in adipose tissue of *ob*$^{-/-}$ mice. Leptin was administered to C57BL-*ob*$^{-/-}$ mice and adipose proteins were extracted at 48 h. Ang2 was determined by immunoblot analysis (50 μg protein/lane) with specific antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). The non-specific band (N.S.) serves for normalization of the immunoblot.

Figure 7 shows induction of Ang2 by leptin in cultured adipocytes. Cultures of undifferentiated mouse 3T3-F442A pre-adipocytes and differentiated adipocytes were induced with leptin (1 μg/ml). Total RNA was extracted at different time points and subjected to RNA blotting with probes to mouse Ang2, VEGF and actin. Notice the punctuate induction of Ang2 in adipocytes at 24 h and the reduction in VEGF level following differentiation of pre-adipocytes into mature adipocytes.

Detailed Description

Recently, leptin was reported to act as an angiogenic factor. It induced human umbilical vein endothelial cell proliferation in vitro, enhanced the formation of capillary-like tubes in vitro and induced neovascularization in corneas of mice and in a chic chorioallantoic membrane (M. R. Sierra-Honigmann, et al., *Science* **281**, 1683-1686, 1998; A. Bouloumie, H. C. Drexler, M. Lafontan, R. Busse, *Circ Res* **83**, 1059-1066, 1998). Although these studies suggested that leptin may induce angiogenesis at its site of production, the role of leptin as an angiogenic factor in the adipose tissue and in tumors has not yet been studied.

It has been found in accordance with the present invention that leptin, optionally together with other agents, acts as inducer of blood vessel regression in tissues and in tumors. Leptin potently induces the expression of the angiostatic factor angiopoietin-2 (Ang2) in various tissues, including adipose tissues and tumors. Ang2 is angiostatic in the absence of VEGF. Thus leptin is effective for modulating angiogenesis, and inhibiting unwanted angiogenesis, especially angiogenesis-related to tumor growth, adipose tissue growth and the estrous cycle.

The present invention includes the use of leptin, or homologues of leptin, or derivatives of leptin, optionally together with one or more inhibitors of VEGF production or VEGF action (hereinafter: "VEGF inhibitors") as inhibitors of tumor angiogenesis and modulators of angiogenesis in the female reproductive organs.

The present invention also includes the use of leptin, or homologues of leptin, or derivatives of leptin, together with VEGF inhibitors to induce adipose-tissue regression and to modulate angiogenesis in the female

reproductive organs.

Administration of leptin, or homologues of leptin, or leptin derivatives, either alone or together with VEGF inhibitors to a human or animal with prevascularized metastasized tumors will prevent the growth or expansion of those tumors.

Administration of leptin, or homologues of leptin, or leptin derivatives, in combination with VEGF inhibitors or other inhibitors of angiogenesis to females will modulate angiogenesis in their reproductive organs.

Diseases and processes that are mediated by angiogenesis include, but are not limited to, hemangioma, solid tumors, blood borne tumors, leukemia, metastasis, telangiectasia, psoriasis, scleroderma, pyogenic granuloma, myocardial angiogenesis, Crohn's disease, plaque neovascularization, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, Helicobacter related diseases, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, placentation, and cat scratch fever.

Administration of leptin, or homologues of leptin, or leptin derivatives, together with VEGF inhibitors or other inhibitors of angiogenesis to human or animal will reduce abberant angiogenesis associated with the aforesaid diseases more effectively than the VEGF inhibitor alone or other inhibitors of angiogenesis when applied without leptin.

It is also possible to modulate angiogenic processes by gene therapy as will be described hereinafter.

The present invention includes the method of treating an

angiogenesis-mediated disease with an effective amount of leptin, or homologues of leptin, or a leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis. The effective amount of leptin, or homologues of leptin, or a leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis is administered to patients in a pharmaceutically acceptable composition.

It is to be understood that the present invention is contemplated to include the use of any homologues of leptin that induce endothelial inhibitory activity. Homologues of leptin refer to proteins, in which one or more of the amino acid residues of a natural leptin are replaced by different amino acid residues, or are deleted, or one or more amino acid residues are added to the natural sequence of leptin, without changing considerably the activity of the resulting products as compared with the wild type leptin. These homologues are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable therefor.

Any such homologue preferably has a sequence of amino acids sufficiently duplicative of that of leptin, such as to have substantially similar activity to leptin. One such activity is the ability of a leptin homologue to reduce the body weight of ob/ob mice. Thus, it can be determined whether any given homologue has substantially the same activity as leptin by means of routine experimentation.

In a preferred embodiment, any such mutein has at least 40% sequence identity or homology with the sequence of either leptin. More preferably, it has at least 50%, at least 60%, at least 70%, at least 80% or, most preferably, at least 90% sequence identity or homology thereto.

Homologues of leptin polypeptides, which can be used in accordance with

the present invention, or nucleic acid coding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. et al., *Principles of Protein Structure*, Springer-Verlag, New York, 1978; and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel et al, *supra*, at §§ A.1.1-A.1.24, and Sambrook et al, *supra*, at Appendices C and D.

Preferred changes for homologues in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions of leptin polypeptides may include synonymous amino acids within a group which have sufficiently similar physicochemical properties that substitution between members of the group will preserve the biological function of the molecule, Grantham, Science, Vol. 185, pp. 862-864 (1974). It is clear that insertions and deletions of amino acids may also be made in the above-defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, *e.g.*, under thirty, and preferably under ten, and do not remove or displace amino acids which are critical to a functional conformation, *e.g.*, cysteine residues, Anfinsen, "Principles That Govern The Folding of Protein Chains", Science, Vol. 181, pp. 223-230 (1973). Proteins and muteins produced by such deletions and/or insertions come within the purview of the present invention.

Preferably, the synonymous amino acid groups are those defined in Table

I. More preferably, the synonymous amino acid groups are those defined in Table II; and most preferably the synonymous amino acid groups are those defined in Table III.

TABLE I

Preferred Groups of Synonymous Amino Acids

Amino Acid	Synonymous Group
Ser	Ser, Thr, Gly, Asn
Arg	Arg, Gln, Lys, Glu, His
Leu	Ile, Phe, Tyr, Met, Val, Leu
Pro	Gly, Ala, Thr, Pro
Thr	Pro, Ser, Ala, Gly, His, Gln, Thr
Ala	Gly, Thr, Pro, Ala
Val	Met, Tyr, Phe, Ile, Leu, Val
Gly	Ala, Thr, Pro, Ser, Gly
Ile	Met, Tyr, Phe, Val, Leu, Ile
Phe	Trp, Met, Tyr, Ile, Val, Leu, Phe
Tyr	Trp, Met, Phe, Ile, Val, Leu, Tyr
Cys	Ser, Thr, Cys
His	Glu, Lys, Gln, Thr, Arg, His
Gln	Glu, Lys, Asn, His, Thr, Arg, Gln
Asn	Gln, Asp, Ser, Asn
Lys	Glu, Gln, His, Arg, Lys
Asp	Glu, Asn, Asp
Glu	Asp, Lys, Asn, Gln, His, Arg, Glu
Met	Phe, Ile, Val, Leu, Met
Trp	Trp

TABLE II

More Preferred Groups of Synonymous Amino Acids

Amino Acid	Synonymous Group
Ser	Ser
Arg	His, Lys, Arg
Leu	Leu, Ile, Phe, Met
Pro	Ala, Pro
Thr	Thr
Ala	Pro, Ala
Val	Val, Met, Ile
Gly	Gly
Ile	Ile, Met, Phe, Val, Leu
Phe	Met, Tyr, Ile, Leu, Phe
Tyr	Phe, Tyr
Cys	Cys, Ser
His	His, Gln, Arg
Gln	Glu, Gln, His
Asn	Asp, Asn
Lys	Lys, Arg
Asp	Asp, Asn
Glu	Glu, Gln
Met	Met, Phe, Ile, Val, Leu
Trp	Trp

TABLE III

Most Preferred Groups of Synonymous Amino Acids

Amino Acid	Synonymous Group
Ser	Ser
Arg	Arg
Leu	Leu, Ile, Met
Pro	Pro
Thr	Thr
Ala	Ala
Val	Val
Gly	Gly
Ile	Ile, Met, Leu
Phe	Phe
Tyr	Tyr
Cys	Cys, Ser
His	His
Gln	Gln
Asn	Asn
Lys	Lys
Asp	Asp
Glu	Glu
Met	Met, Ile, Leu
Trp	Met

Examples of production of amino acid substitutions in proteins which can be used for obtaining homologues of leptin polypeptides or proteins for use in the

present invention include any known method steps, such as presented in US patents RE 33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Koths et al., 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and lysine substituted proteins presented in US patent No. 4,904,584 (Shaw et al).

In another preferred embodiment of the present invention, any homologue of leptin has an amino acid sequence essentially corresponding to that of leptin. The term "essentially corresponding to" is intended to comprehend proteins with minor changes to the sequence of the natural protein which do not affect the basic characteristics of the natural proteins, particularly insofar as their ability to induce angiostatic activity. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding these proteins, resulting in a few minor modifications, and screening for the desired activity in the manner discussed above.

It is to be understood that the present invention is contemplated to include the use of any derivatives of leptin that induce endothelial inhibitory activity when applied optionally together with a VEGF inhibitor or other inhibitors of angiogenesis. The present invention includes the use of an entire leptin protein, the use of derivatives of the leptin protein and the use of biologically active fragments of the leptin protein. Derivatives of leptin according to the invention have one or more chemical moieties attached thereto, including water-soluble polymers such as polyethylene glycol. Polyethylene glycol derivatized derivatives can be mono-, di-, tri- or tetrapegylated e. g., N-terminal monopegylated. Preferred N-terminal monopeglyated derivatives of leptin, optionally having a (pegylated) methionine at the N-terminus.

Various inhibitors of VEGF activity or VEGF production have been described and may be used in combination with leptin in order to inhibit angiogenesis. Among these inhibitors are 3,7-dimethyl-1-propargylxanthine(DMPX), an A2-antagonist, 7-(beta-hydroxyethyl)theophylline, 8-phenyltheophylline, the adenosine A2 receptor antagonist CSC (8-(3-chlorostyryl)caffeine), theobromine, an antagonistic VEGF variant, the soluble VEGF receptor sFLT-1, Tranilast, 8-(3-oxo-4,5,6-trihydroxy-3h-xanthen-9-yl)-1-naphthoic acid, suramin and platelet factor-4 (E. Hashimoto, et al., *Biochem Biophys Res Commun* **204**, 318-24, 1994; S. Fischer, R. Knoll, D. Renz, G. F. Karliczek, W. Schaper, *Endothelium* **5**, 155-165, 1997; H. Takagi, G. L. King, G. S. Robinson, N. Ferrara, L. P. Aiello, *Invest Ophthalmol Vis Sci* **37**, 2165-2176, 1996; E. Barcz, et al., *Oncol Rep* **5**, 517-520, 1998; G. Siemeister, et al., *Proc Natl Acad Sci U S A* **95**, 4625-4629, 1998; W. Roeckl, et al., *Exp Cell Res* **241**, 161-170, 1998, S. Komaya et al., *Br J Pharmacol* **127**, 537-545, 1999: K. Igarashi et al., *Int J Mol Med* **2**, 211-215, 1998; J. Waltenberger et al., *J Mol Cell Cardiol* **28**, 1523-1529, 1996; S. Grengrinovitch et al., *J Biol Chem* **270**, 15059-15065, 1995).

Various inhibitors of angiogenesis have been described and may be used in combination with leptin in order to inhibit angiogenesis more effectively than when used alone. Among these inhibitors are K1-5 (Cao, R. et al, Proc Natl Acad Sci U S A, 96, 5728-5733, 1999), angiostatin, endostatin, BB-94 and AGM-1470 (Bergers G. et al, Science284, 808-812, 1999).

Also comprised by the present invention is the use of expression vectors encoding leptin or leptin homologues, provided by gene therapy, optionally together with inhibitors of VEGF action or production or other inhibitors of angiogenesis for inhibition of angiogenesis in tumors. Such medicaments can be

employed in therapeutic methods involving intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, nasal, oral or pulmonary delivery systems.

Also comprised by the present invention is the use of expression vectors encoding leptin or leptin homologues, provided by gene therapy, in combination with inhibitors of VEGF action or production or other inhibitors of angiogenesis for regression of adipose tissues. Such therapy may be useful in treating a disorder selected from the group consisting of diabetes, high blood pressure and high cholesterol and as part of combinative therapy with a medicament for treating such disorders. Such medicaments can be employed in therapeutic methods involving intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, nasal, oral or pulmonary delivery systems.

The angiogenesis mediated diseases include, but are not limited to, obesity; solid tumors; blood born tumors such as leukemias; tumor metastasis; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints, angiofibroma; and wound granulation.

Leptin, or homologues of leptin, or leptin derivatives, optionally together with VEGF or other inhibitors of angiogenesis inhibitors are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, Crohn's disease, arteriosclerosis, scleroderma, and hypertrophic scars, i.e., keloids.

Leptin, or homologues of leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with leptin, or homologues of leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis and then leptin, or homologues of leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

Additionally, Leptin, or homologues of leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis, are combined with pharmaceutically acceptable excipients. Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Compositions may optionally include sustained-release matrix, such as biodegradable polymers, to form therapeutic compositions. A sustained-release

matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid/base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. The sustained-release matrix desirably is chosen from biocompatible materials such as liposomes, polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (co-polymers of lactic acid and glycolic acid) polyanhydrides, poly(ortho)esters, polypeptides, hyaluronic acid, collagen, chondroitin sulfate, carboxylic acids, fatty acids, phospholipids, polysaccharides, nucleic acids, polyamino acids, amino acids such as phenylalanine, tyrosine, isoleucine, polynucleotides and polyvinylpyrrolidone.

A preferred biodegradable matrix is a matrix of one of either polylactide, polyglycolide, or polylactide co-glycolide (co-polymers of lactic acid and glycolic acid). The polymers being implanted in the vicinity of where drug delivery is desired, for example, at the adipose tissue or at a site of a tumor or implanted, so that the leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem et al., J. Neurosurg. 74:441-446 (1991), which is hereby incorporated by reference in its entirety.

The angiogenesis-modulating pharmaceutical compositions according to the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid therapeutic compositions include pills, creams, and implantable dosage units. The pills may be administered orally; the therapeutic creams may be administered topically. The implantable dosage units may be administered locally, for example at a tumor site, or may be implanted for systemic release of the therapeutic

angiogenesis-modulating composition, for example subcutaneously. Examples of liquid compositions include compositions adapted for injection subcutaneously, intravenously, intraarterially, and compositions for topical and intraocular administration. Examples of aerosol compositions include inhaler composition for administration to the lungs.

It should be understood that in addition to the ingredients, specifically mentioned above, the compositions according to the present invention may include other agents conventional in the art having regard to the type of composition in question. Optionally, cytotoxic agents may be incorporated or otherwise combined with leptin, or homologues of leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis, to provide dual therapy to the patient.

The compositions according to the invention can be administered by standard routes. In general, the combinations may be administered by the topical (including buccal and sublingual), or parenteral (including subcutaneous, intraperitoneal, intramuscular, intravenous, intradermal, intracerebral, intracerebroventricular, intracranial, intraspinal, intratracheal, and epidural), transdermal, intravaginal, intrauterine, oral, rectal, ophthalmic (including intravitreal or intracameral), or intranasal, administration.

Osmotic minipumps may also be used to provide controlled delivery of high concentrations of leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor.

The dosage of the leptin, or leptin derivatives, optionally together with VEGF inhibitors or other inhibitors of angiogenesis of the present invention will

depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating humans or animals, between approximately 0.5 mg/kilogram to 10 mg/kilogram of the leptin or leptin homologue or leptin derivative can be administered, optionally together with a suitable dose of a VEGF inhibitor or inhibitor of VEGF production or other inhibitors of angiogenesis. Depending upon the half-life of the leptin or leptin homologue or leptin derivative in the particular animal or human, the leptin or leptin homologue or leptin derivative can be administered between several times per day to once a week. Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The leptin or leptin homologue or leptin derivative compositions may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, specifically mentioned above, the compositions of the present invention may include other agents conventional in the art having regard to the type of composition in

question.

It is to be understood that the present invention has application for both human and veterinary use.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

Examples

Example 1

Induction of blood vessel regression by leptin

To test leptin's effect on blood vessel homeostasis in adult adipose tissue, murine leptin (0.1-5 μg/g) was injected ip at time 0 and 9 h to 8-10 weeks old obese (C57BL-ob$^{-/-}$) female mice, lacking endogenous leptin. A noticeable weight loss was observed at 48 h in mice receiving ≥ 2x1 μg/g leptin (65.4±0.5 g vs. 62.7±1.0 g, n=6). Abdominal fat was removed and fixed 24 and 48 h after the first injection, and blood vessels were counted after staining paraffin sections with antibodies to Factor VIII (D.D. Wagner et al. J Cell Biol 95, 355-360 (1982)). A significant reduction in the number of blood vessels was observed (198±1 vessels per 5 high power fields (HPFs, x400) in control mice; 159±2.5 vessels per 5 HPFs in leptin-treated mice (2x1 μg/g) at 24 h and 106±7.5 vessels per 5 HPFs at 48 h. **Figure 1** shows micrographs of blood vessels in adipose

tissue sections. **Figure 2** shows the dose-response curve of the blood vessel regression and **Figure 3** shows the time course of this regression.

Example 2

Leptin induces Angiopoietin 2 (Ang2) in adipose tissues

The mechanism by which leptin induced the blood vessel regression in adipose tissues was studied by measuring its effect on the expression level of angiogenic and angiostatic factors. Total RNA was isolated from adipose tissue of C57BL and C57BL-*ob*$^{-/-}$ mice at time 0 and 24 h after the first leptin administration. Total RNA was isolated with the TRI reagent. Reverse transcription was carried out in 20 µl volume using RNase H$^-$ reverse transcriptase (SuperScript II, GIBCO-BRL) with 1 µg $(N)_6$ random primer (New England Biolabs) according to the manufacturer's instructions. Aliquot (2 µl) of the reverse transcription product was used for PCR with VENT DNA polymerase (New England Biolabs) and the following sense and antisense primers: muAng-2 mRNA, GeneBank Accession No. AF4326 nucleotides 637-657 and 1147-1167; muVEGF, GeneBank Accession No. M95200 nucleotides 385-406 and 962-980; muActin mRNA, GeneBank Accession No. J00691 nucleotides 1670-1691 and 2452-2431. PCR reactions were terminated before saturation. It was found that Ang2 mRNA is expressed in adipose tissue of normal mice and not in that of the *ob*$^{-/-}$ mice. Furthermore, injection of leptin induced the expression of Ang2 in both types of mice **(Figure 4)**. These results demonstrate that leptin is a potent inducer of the angiostatic factor Ang2.

The levels and induction of Ang2 mRNA by leptin in the adipose tissue of

ob$^{-/-}$ mice was then studied by RNA blotting with specific probes to Ang2 and VEGF. Total RNA from adipose tissue was isolated with the TRI reagent kit (Molecular Research Center Inc.). Samples of RNA (15 µg) were resolved by electrophoresis through 1% agarose gel in MOPS-formaldehyde buffer, transferred to nylon membrane (Hybond N, Amersham) in 20XSSC buffer and the membrane was then heated for 2 hours at 80°C in a vacuum oven. The membrane was pre-hybridized (6 h, 42°C) with denatured Salmon-sperm DNA (100 µg/ml in 50% formamide, 5xSSC, 4xDenhard's solution and 0.5% SDS). A [^{32}P]dCTP DNA probe (1x10^{6} cpm/ml), prepared by random priming, was then added and hybridization continued for 18 hours at 42°C. The membrane was then washed at room temperature (1xSSC, 0.1% SDS twice, 0.25xSSC, 0.1% SDS and 0.1xSSC, 0.1% SDS twice, 30 min. each wash) and autoradiographed. Blots were then re-hybridized with ^{32}P-labeled probe corresponding to mouse actin to show equal amounts of RNA in the blot. A significant induction of Ang2 was obtained following leptin administration (2.9±0.4 fold, $P<0.05$, n=3 at 24 h and 16.0±0.31 fold, $P<0.01$, n=3 at 48 h; **Figure 5**). The kinetics of Ang2 expression corresponded to that of the apoptosis and blood vessel regression. The level of VEGF mRNA was only slightly induced (1.4±0.1 fold, n=3 at 48 h; **Figure 5**).

The dose-response of leptin-induced Ang2 in adipose tissues was studied by immunoblotting 48 h after the first injection of leptin. Cell extracts from adipose tissue were isolated using the TRI reagent kit (Molecular Research Center Inc.) in parallel to total RNA extraction. Fifty micrograms protein were separated on 10% SDS-polyacrylamide gel. Immunoblot analysis was carried out with 5 µg of a specific goat anti-human Ang-2 antibody. Ang2 was below the level of detection in adipose tissue of control *ob*$^{-/-}$ mice, whereas administration

of 2x1 μg/g leptin was sufficient for high level induction of Ang2 (**Figure 6**).

Example 3

Leptin induces Angiopoietin 2 (Ang2) in cultured adipocytes

Several peripheral activities of leptin were previously reported (M. R. Sierra-Honigmann, et al., *Science* **281**, 1683-1686, 1998; A. Bouloumie, H. C. Drexler, M. Lafontan, R. Busse, *Circ Res* **83**, 1059-1066,1998; B. Cohen, D. Novick, M. Rubinstein, *Science* **274**, 1185-1188, 1996; D. Barkan, et al., *Endocrinology* **140**, 1731-1738, 1999). To test if leptin may act directly on adipocytes, we studied the effect of leptin on murine 3T3-F442A pre-adipocytes, known to give rise to adipose-like tissue upon implantation in athymic mice (H. Green, O. Kehinde, *J Cell Physiol* **101**, 169-171, 1979); S. Mandrup, T. M. Loftus, O. A. MacDougald, F. P. Kuhajda, M. D. Lane, *Proc Natl Acad Sci U S A* **94**, 4300-4305,1997). Swiss 3T3 F442A murine pre-adiopcytes (H. Green, O. Kehinde, *Cell* **5**, 19-27, 1975) were grown in DMEM (GIBCO) and 10% calf serum. For differentiation, confluent cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) for six days. Medium was replaced every 48 hours. By the end of the period most of the cells acquired the characteristic adipocyte morphology as determined by biochemical and morphological criteria. Leptin (1 μg/ml) was added to cultures of differentiated and non-differentiated cells. RNA was isolated from the cultured cells as described for the adipose tissues of Example 2 and subjected to RNA blot analysis. It was found that leptin induced Ang2 mRNA expression in differentiated 3T3-F442A adipocytes and not in pre-adipocytes. Ang2 mRNA appeared punctuate at 24 h. VEGF mRNA was constitutively expressed in

pre-adipocytes and was further induced by leptin. The level of VEGF mRNA was significantly lower in mature adipocytes and was not significantly induced by leptin **(Figure 7).** These result suggest that leptin induces an angiostatic signal in mature adipocytes and angiogenic signals in pre-adipocytes.

Example 4

Effect of leptin plus a VEGF inhibitor on adipose mass reduction

The angiostatic activity of leptin-induced Ang2 is reversed in the presence of VEGF. Furthermore, a modest induction of VEGF by leptin was noticed in the previous examples. Therefore, murine leptin (0.1-5 μg/g) is injected ip at time 0 and 9 h to 8-10 weeks old obese (C57BL-ob$^{-/-}$) female mice, lacking endogenous leptin. In parallel, 8-10 weeks old obese (C57BL-ob$^{-/-}$) female mice were injected at times 0 and 9 h ip with murine leptin (0.1-5 μg/g) together with the adenosine 2 receptor antagonist CSC, known to function as a VEGF inhibitor (H. Takagi, G. L. King, G. S. Robinson, N. Ferrara, L. P. Aiello, *Invest Ophthalmol Vis Sci* **37**, 2165-2176, 1996). A noticeable weight loss was observed at 48 h in mice receiving ≥ 2x1 μg/g leptin alone (65.4±0.5 g vs. 62.7±1.0 g, n=6). A significantly higher weight loss is observed in mice treated with a combination of leptin and CSC.

It should be understood that the foregoing relates only to preferred embodiments of the present invention, and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

CLAIMS:

1. The use of leptin or a leptin homologue or derivative optionally together with an inhibitor of VEGF action or of VEGF synthesis and/or an inhibitor of angiogenesis, in the preparation of a medicament reversibly inhibiting endothelial cell proliferation.

2. The use according to claim 1 for modulating angiogenic processes.

3. The use according to claim 2 for inhibiting angiogenesis.

4. The use according to claim 3, including an angiogenesis inhibitor.

5. The use according to anyone of the preceding claims wherein the VEGF inhibitor is selected from DMPX, an A2-antagonist 7-(betahydroxyethyl)theophylline, 8-phenyltheophylline, the adenosine A2 receptor antagonist CSC, theobromine, an antagonistic VEGF variant, sFLT-1, Tranilast, 8-(3-oxo-4,5,6-trihydroxy-3h-xanthen-9-yl)-1-naphthoic acid, suramin and platelet factor-4 .

6. A pharmaceutical composition for reversibly inhibiting endothelial cell proliferation comprising leptin or a leptin homologue or derivative optionally together with an inhibitor of VEGF action or of VEGF synthesis and/or an inhibitor of angiogenesis.

7. A pharmaceutical composition according to claim 6 for modulating

angiogenic processes.

8. A pharmaceutical composition according to claim 7 for inhibiting angiogenesis.

9. A method for reversibly inhibiting endothelial cell proliferation in mammals comprising administering to a subject a pharmaceutical composition according to any one of claims 6 to 9 in a suitable dosage form and by a suitable route of administration.

10. A mixture comprising leptin and a VEGF inhibitor.

ABSTRACT

Disclosed is the use of leptin, optionally together with VEGF inhibitors, in inhibition of endothelial cell proliferation and modulation of angiogenesis.

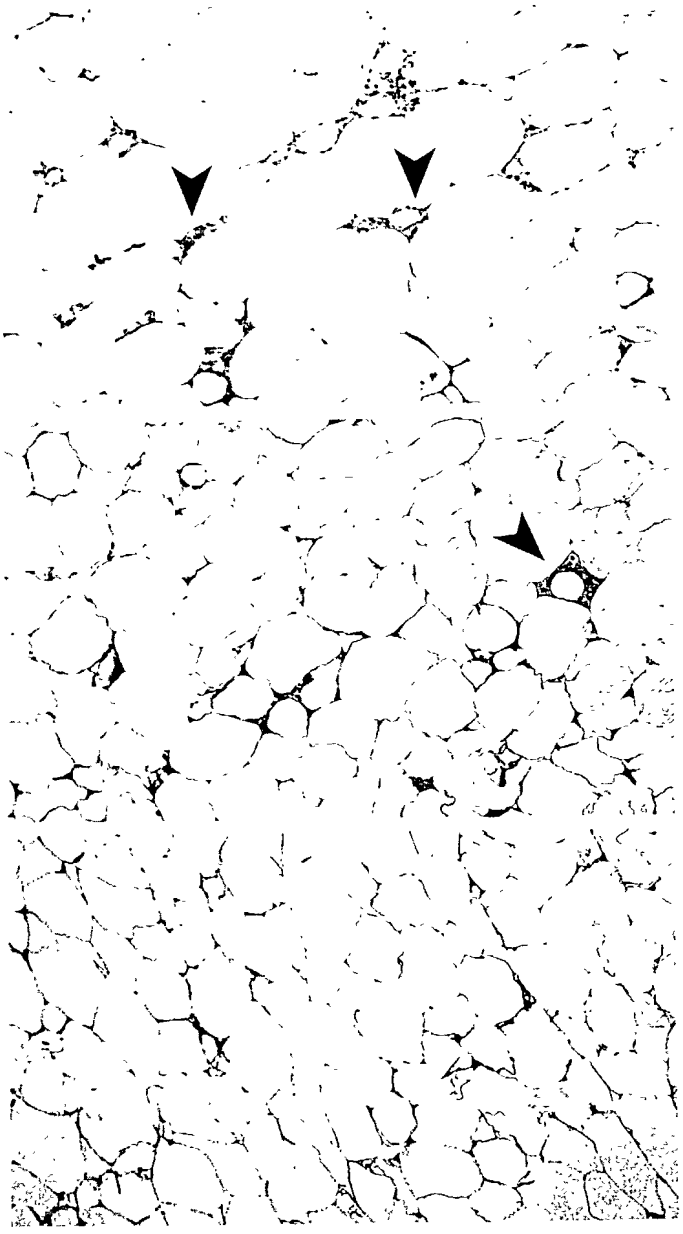

FIGURE 1

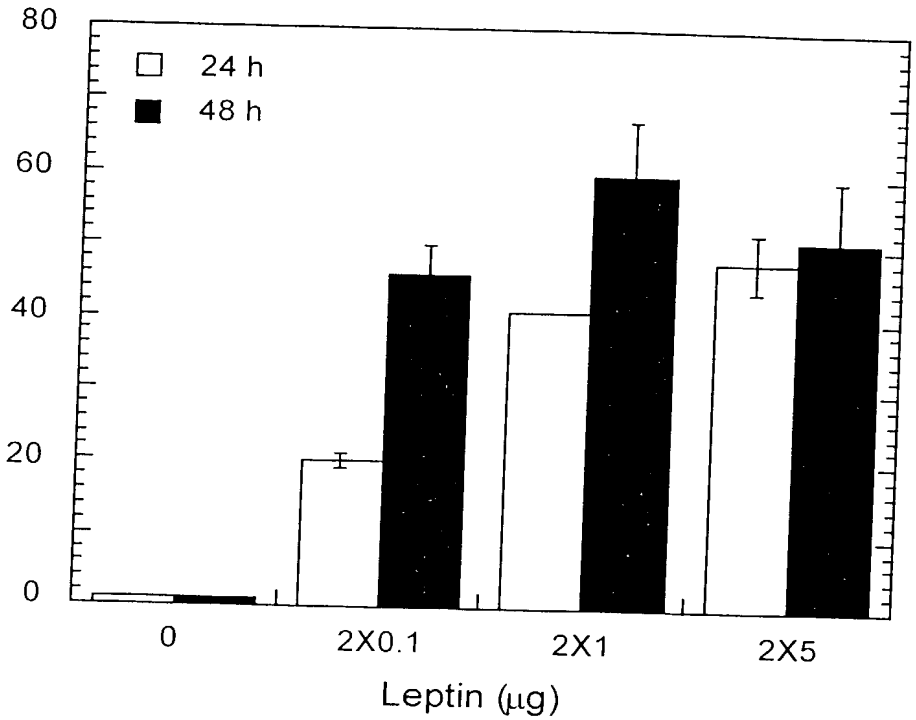

FIGURE 2

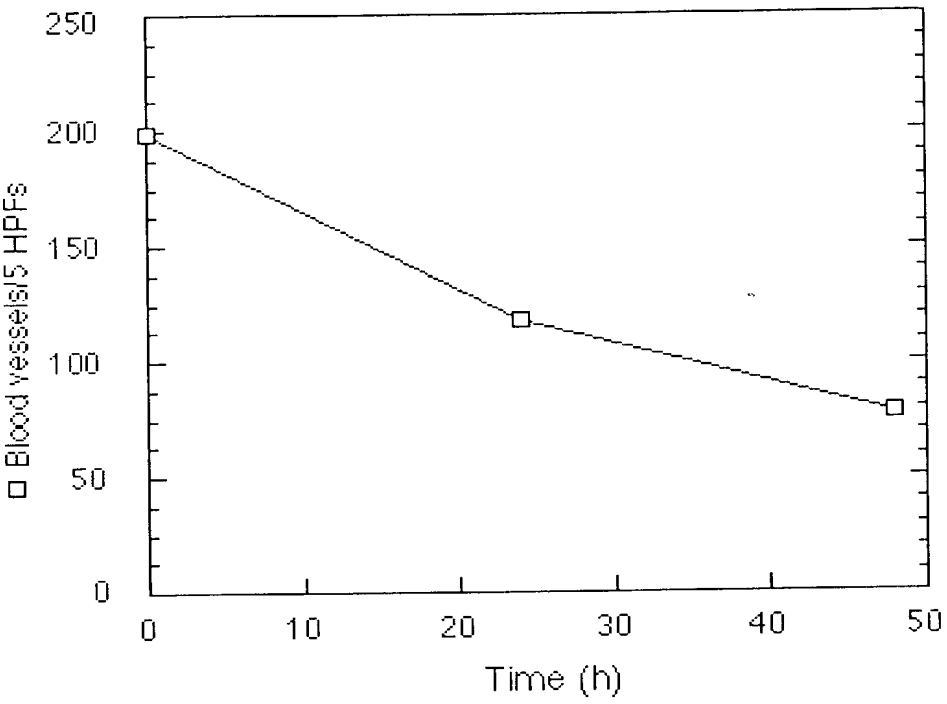

FIGURE 3

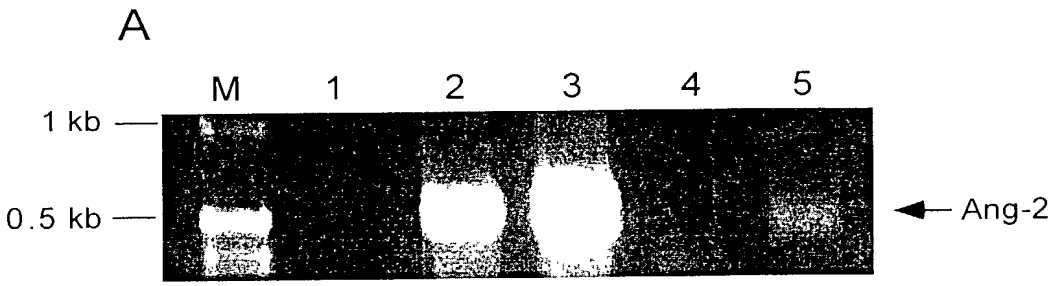

FIGURE 4

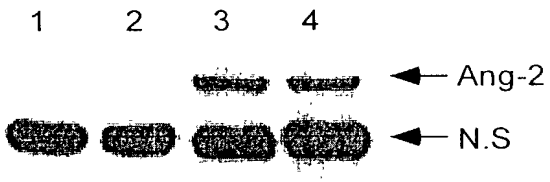

FIGURE 6

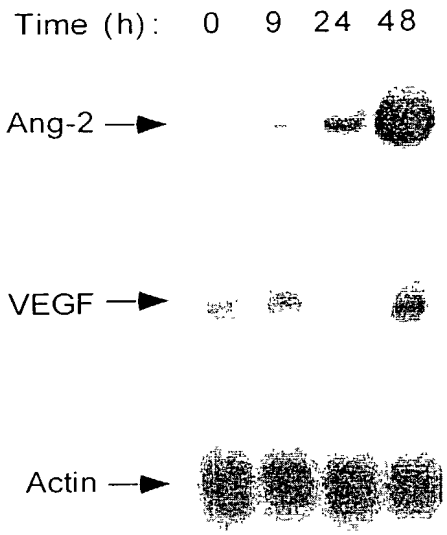

FIGURE 5

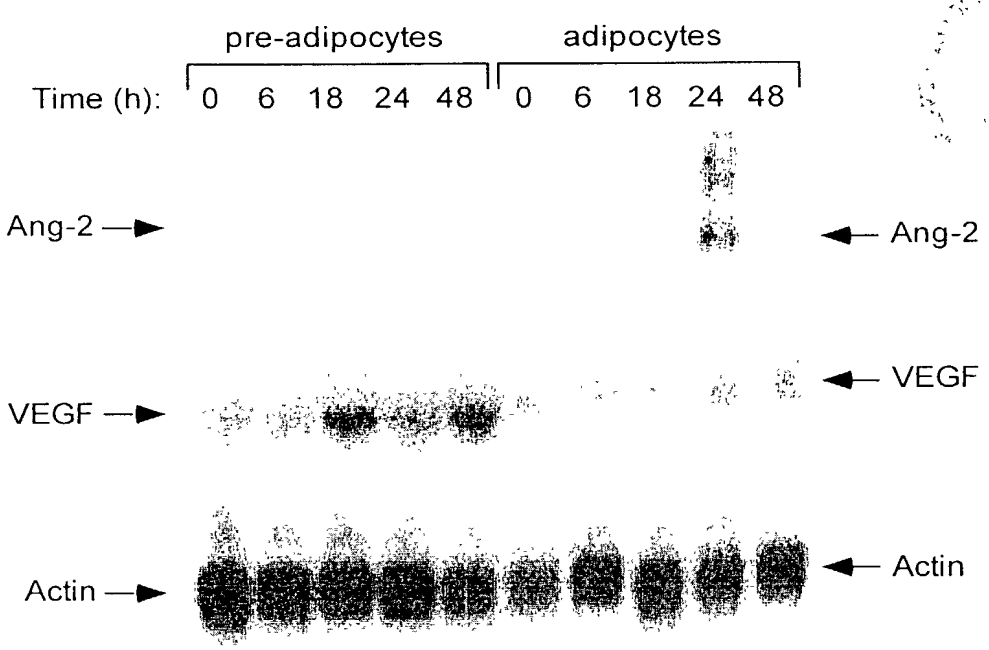
pre-adipocytes
adipocytes
Time (h):
0 6 18 24 48 0 6 18 24 48
Ang-2
Ang-2
VEGF
VEGF
Actin
Actin

FIGURE 7

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10070295 .090902

#5

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APPLICATION INFORMATION

Title Line One:: USE OF LEPTIN IN INHIBITION OF ENDOTHELI
Title Line Two:: AL CELL PROLIFERATION
Total Drawing Sheets:: 7
Formal Drawings?:: Yes
Docket Number:: RUBINSTEIN=7
Secrecy Order in Parent Appl.?:: No

REPRESENTATIVE INFORMATION

Representative Customer Number:: 1444

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> Application One:: PCT/IL00/00525
Filing Date:: 09-04-2000

PRIOR FOREIGN APPLICATIONS

Foreign Application One:: 131739
Filing Date:: 09-05-1999
Country:: Israel
Priority Claimed:: Yes
Foreign Application Two:: 132312
Filing Date:: 10-10-1999
Country:: Israel
Priority Claimed:: Yes

Source:: PrintEFS Version 1.0.1

10070295 .090902 #3

Page 1 of 2 Pages [X] Original [ ] Substitute [ ] Supplemental Atty. Docket: RUNBINSTEIN=7

Combined Declaration for Patent Application and Power of Attorney

As a below-named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name, and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

USE OF LEPTIN IN INHIBITION OF ENDOTHELIAL CELL PROLIFERATION

the specification of which (check one)

[ ] is attached hereto;
[ ] was filed in the United States under 35 U.S.C. §111 on , as U S Appln No _____ *, or
[X] was/will be filed in the U S under 35 U S C §371 by entry into the U S national stage of an international (PCT) application, PCT/IL00/005625; filed September 4, 2000, entry requested on March 5, 2002*, national stage application received U S Appln No _____*, §371/§102(e) date _____* (* if known)

and was amended on (if applicable)
(include dates of amendments under PCT Art 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above, and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C §§ 119 (a)-(d) and 365 (b) of any prior foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or under §365(a) of any PCT application which designated at least one country other than the U S , listed below

Application No	Country	Filing Date (MM/DD/YYYY)
131739	Israel	09-05-1999
132312	Israel	10-10-1999

If I claimed foreign priority above, I hereby identify below any foreign application for patent (including an international (PCT) application designating a country other than the United States) or for an inventor's or plant breeder's certificate, having a filing date before that of the earliest application from which foreign priority is claimed (if left blank, then there are none):

Non-Priority Application No.	Country	Filing Date (MM/DD/YYYY)

I hereby claim the benefit under 35 U S C §119(e) of any United States provisional applications listed below:

Application No	Filing Date (MM/DD/YYYY)

I hereby claim the benefit under 35 U S C §120 of any prior U S non-provisional application(s) or under §365(c) of any prior PCT international application(s) designating the U S , listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U S or PCT international application in the manner provided by the first paragraph of 35 U.S.C §112, I acknowledge the duty to disclose to the PTO all information which is material to patentability as defined in 37 C.F.R §1 56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Application No.	Filing Date (MM/DD/YYYY)	Status (patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

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Page 2 of 2 Pages Atty Docket: RUBINSTEIN=7

Title: USE OF LEPTIN IN INHIBITION OF ENDOTHELIAL CELL PROLIFERATION

U.S. Application filed _____, Serial No. _____

PCT Application filed _____, Serial No _____

The undersigned hereby authorizes the U S Attorneys or Agents appointed herein to accept and follow instructions from Serono as to any action to be taken in the U S Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U S Attorneys or Agents appointed herein will be so notified by the undersigned

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U S C §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

1-00

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FULL NAME OF THIRD JOINT INVENTOR: Dalit BARKAN	INVENTOR'S SIGNATURE	DATE
RESIDENCE: Rehovot, Israel		CITIZENSHIP: Israel
POST OFFICE ADDRESS: 22/A Vilkomitch St., 76448 Rehovot, Israel		
FULL NAME OF FOURTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.

Page 1 of 2 Pages [X] Original [ ] Substitute [ ] Supplemental Atty. Docket: RUNBINSTEIN=7

Combined Declaration for Patent Application and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

USE OF LEPTIN IN INHIBITION OF ENDOTHELIAL CELL PROLIFERATION

the specification of which (check one)

[ ] is attached hereto,

[ ] was filed in the United States under 35 U.S.C §111 on , as U S Appln. No. _____ *; or

[X] was/will be filed in the U.S. under 35 U.S.C §371 by entry into the U.S national stage of an international (PCT) application, PCT/IL00/005625; filed September 4, 2000, entry requested on March 5, 2002*, national stage application received U.S. Appln No. _____*; §371/§102(e) date _____* (* if known)

and was amended on (if applicable).
(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above, and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1 56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 (a)-(d) and 365 (b) of any prior foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or under §365(a) of any PCT application which designated at least one country other than the U.S., listed below:

Application No.	Country	Filing Date (MM/DD/YYYY)
131739	Israel	09-05-1999
132312	Israel	10-10-1999

If I claimed foreign priority above, I hereby identify below any foreign application for patent (including an international (PCT) application designating a country other than the United States) or for an inventor's or plant breeder's certificate, having a filing date before that of the earliest application from which foreign priority is claimed (if left blank, then there are none).

Non-Priority Application No.	Country	Filing Date (MM/DD/YYYY)

I hereby claim the benefit under 35 U.S.C §119(e) of any United States provisional applications listed below:

Application No	Filing Date (MM/DD/YYYY)

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or under §365(c) of any prior PCT international application(s) designating the U.S., listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U S or PCT international application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information which is material to patentability as defined in 37 C.F.R. §1 56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date (MM/DD/YYYY)	Status (patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

All of the practitioners associated with Customer Number 001444

Direct all correspondence to the address associated with **Customer Number 001444**, which is presently:

BROWDY AND NEIMARK, P.L.L.C.
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Page 2 of 2 Pages

Atty Docket: RUBINSTEIN=7

Title: USE OF LEPTIN IN INHIBITION OF ENDOTHELIAL CELL PROLIFERATION

U.S. Application filed _____, Serial No _____

PCT Application filed _____, Serial No _____

The undersigned hereby authorizes the U S Attorneys or Agents appointed herein to accept and follow instructions from Serono as to any action to be taken in the U S. Patent and Trademark Office regarding this application without direct communication between the U S Attorneys or Agents and the undersigned In the event of a change of the persons from whom instructions may be taken, the U S Attorneys or Agents appointed herein will be so notified by the undersigned

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U S C §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

FULL NAME OF FIRST INVENTOR	INVENTOR'S SIGNATURE	DATE
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